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Gamete Spawning and Fertilization in the Gymnolaemate Bryozoan *Membranipora membranacea*

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Abstract. The simultaneously hermaphroditic zooids of *Membranipora membranacea* colonies spawn primary oocytes and spermatozeugmata (aggregates of 32 or 64 spermatozoa) into ambient seawater. Eggs are released through the intertentacular organ (ITO) whereas spermatozeugmata are spawned through the tips of the two distomedial tentacles. Fertilized eggs undergo planktotrophic development to form long-lived cyphonautes larvae. Examination of ovarian, coelomic, and spawned oocytes for sperm nuclei, using either Bisbenzimide H33342 or aceto-orcein staining, revealed that a single sperm fuses with primary oocytes during or shortly after ovulation. In *M. membranacea*, egg activation does not immediately follow sperm-egg fusion, but occurs after oocytes are spawned through the ITO. The period between sperm-egg fusion and egg activation may last up to four days. Once zygotes begin to develop, they follow an *Ascaris*-type of fertilization pattern (Wilson, 1925). Internal sperm-egg fusion does not preclude cross-fertilization in *M. membranacea*, because spawned spermatozeugmata enter maternal coeloms through ITOs after being drawn into lophophores. The ITO actively regulates the entrance of spermatozeugmata and the release of oocytes by the closure of the distal pore. The ITO does not act as a filter to prevent self-fertilization, so that the paternal colony may also function as the maternal colony. Self-fertilization may be reduced in *M. membranacea* via increasing sperm dispersal away from the paternal colony, which is accomplished by the bending of the distomedial tentacles such that they release spermatozeugmata into the exhalent feeding current of the colony.

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Abbreviation: ITO—Intertentacular organ.

Introduction

In 1966, Silén reported that external cross-fertilization occurred in two gymnolaemate bryozoan species, *Electra posidoniae* and *E. crustulenta*. This report contradicted the 100-year-old belief that internal self-fertilization is obligatory in this group of hermaphroditic invertebrates (Huxley, 1856; Prouho, 1892; Calvet, 1900; Pace, 1906; Bonnevie, 1907; Marcus, 1938, 1941; Silén, 1944; Correa, 1948; Mawatari, 1952). The two species that Silén (1966) examined have planktotrophic larvae and spawn primary oocytes through a secondary reproductive structure called an intertentacular organ (ITO). The ITO, which is present in several gymnolaemate genera (*e.g., Alcyonidium, Conopeum, Electra, Farella,* and *Membranipora*), forms at the base of the two distomedial tentacles and provides a conduit for oocytes from the maternal visceral coelom to the external seawater. For *E. posidoniae,* Silén reported that spawned sperm caught by lophophores of neighboring colonies became attached to the abfrontal sides of the tentacles. According to Silén, sperm detached from the tentacles and swarmed around oocytes emerging from the ITO. For *E. crustulenta,* Silén was unable to follow sperm, however he reported finding sperm inside the ITO. Silén concluded that fertilization in *E. posidoniae* and *E. crustulenta* is external to the maternal coelom, occurring while oocytes are in either the ITO or seawater. However, Silén did not actually observe sperm-egg fusion in either species of *Electra.*

Silén (1966) proposed that external cross-fertilization may be a general phenomenon among gymnolaemate bryozoans and not restricted to species that possess an ITO. This proposal appears to be supported by two facts. First, spawning of sperm is widespread among bryozoan species. Silén (1966, 1972) and Bullivant (1967) described sperm spawning in 13 gymnolaemate genera. In some

genera, such as *Schizoporella*, sperm are spawned through the tips of all the tentacles, whereas in other genera, such as *Electra* and *Membranipora*, sperm or tightly organized aggregates of 32 or 64 sperm (Fig. 1) called spermatozeugmata (Bonnevie, 1907; Franzén, 1956; Zimmer and Woollacott, 1974), are released through only the two distomedial tentacles. Both Silén (1966, 1972) and Bullivant (1967) suggested that in these species, spawned sperm may externally cross-fertilize eggs as they emerge from the ITO or as they are transferred to an external site for brooding. The second fact supporting Silén's proposal is that egg activation (*e.g.*, elevation of a fertilization envelope), which closely follows sperm-egg fusion in most animal species (*e.g.*, Longo, 1987; Epel, 1990), does not seem to occur in gymnolaemate oocytes until after they are spawned (*e.g.*, Prouho, 1892; Calvet, 1900; Silén, 1966; Reed, 1988, 1991).

To date no study has directly evaluated the proposal that gymnolaemate bryozoans use external cross-fertilization. The lack of such an evaluation is due to the fact that the fertilization process has never been completely described for any gymnolaemate species. I report here that the site of sperm-egg fusion in the planktotrophic species *Membranipora membranacea* is within the maternal coelom, whereas the site of egg activation is in the external seawater. In *M. membranacea*, sperm-egg fusion and egg activation may be temporally separated by as many as four days. Although sperm-egg fusion occurs inside the maternal zooid, cross-fertilization is still possible because spawned spermatozeugmata gain access to the maternal visceral coelom via the ITO.

Materials and Methods

Observations of gamete spawning and egg activation

Observations of gamete spawning and spermatozeugmata transfer were made using one-zooid-row preparations. To make these preparations, sexually reproductive *Membranipora membranacea* colonies growing on *Iridaea cordata* were collected from the surface waters at or near the Friday Harbor Laboratories, University of Washington, San Juan Island, Washington. Colonies encrusting this substrate had two advantages over colonies growing on brown macroalgae, such as *Nereocystis luetkeana*. First, *M. membranacea* population densities on *I. cordata* were low; consequently, colonies several centimeters in diameter were frequently found. Such large colonies often have areas in which the zooids form long, unbranching, and parallel rows. By cutting down alternating rows with a scalpel blade, portions of colonies were made into strips, one-zooid wide and several zooids long. Although zooids on either side of this row were destroyed, the zooids within the row continued to feed and spawn gametes. The second advantage in using *I. cordata* is that after this alga is cut, it secretes a minimal amount of mucus as compared to the brown macroalgae. After cutting the colony into strips, one-zooid rows were rinsed several times with seawater to remove debris and placed in small plastic petri dishes filled with unfiltered seawater. These petri dishes were kept in an incubator maintained at 15°C.

One-zooid rows were placed on their sides in petri dishes. A Zeiss RA 16 Research microscope fitted with a phototube and a Panasonic WV-BL204 black and white television camera was used to observe and record gamete spawning and spermatozeugmata transfer. Images were recorded using a Panasonic VHS video recorder. Selected images were transferred to 35-mm format by photographing frozen frames from a television monitor onto Plus-X panchromatic film.

Two sets of measurements were made using one-zooid-row preparations to determine the length of time for oocytes to be spawned and undergo egg activation. Video images were used to measure the lengths of time for oocytes to pass from the visceral coelom, through the ITO, and into the ambient seawater. For the second set of measurements, a Wild M5 dissecting microscope was used to follow oocytes produced by individuals in one-zooid-row preparations from the time they completed their entry into the ITO until they initiated egg activation. Egg activation was considered to begin when the fertilization envelope first started to elevate. Elapsed time was measured using a digital stop watch.

The ability of seawater to stimulate egg activation in *M. membranacea* oocytes was investigated by removing oocytes from maternal coeloms by dissection and rinsing them with seawater. Oocytes from several zooids were pooled and then distributed into two groups. In one group, oocytes were washed with three exchanges of 0.2 μm filtered seawater, while oocytes in the second group were washed with three exchanges of filtered seawater containing 0.1 m*M* disodium ethylenediamine tetraacetic acid (EDTA). The presence of 0.1 m*M* EDTA enhances the percentage of spawned oocytes that develop in the laboratory (Reed, 1987). Oocytes were used from adjacent sister zooids of the same colony for each trial. The number of oocytes that underwent egg activation and the number of activated oocytes that developed to a swimming gastrula stage were determined for each group.

Observations of fertilization

The events of fertilization were determined from the study of whole mount preparations of ovarian, coelomic, and recently spawned oocytes. Some colonies were fixed before ovaries and coelomic oocytes were removed from

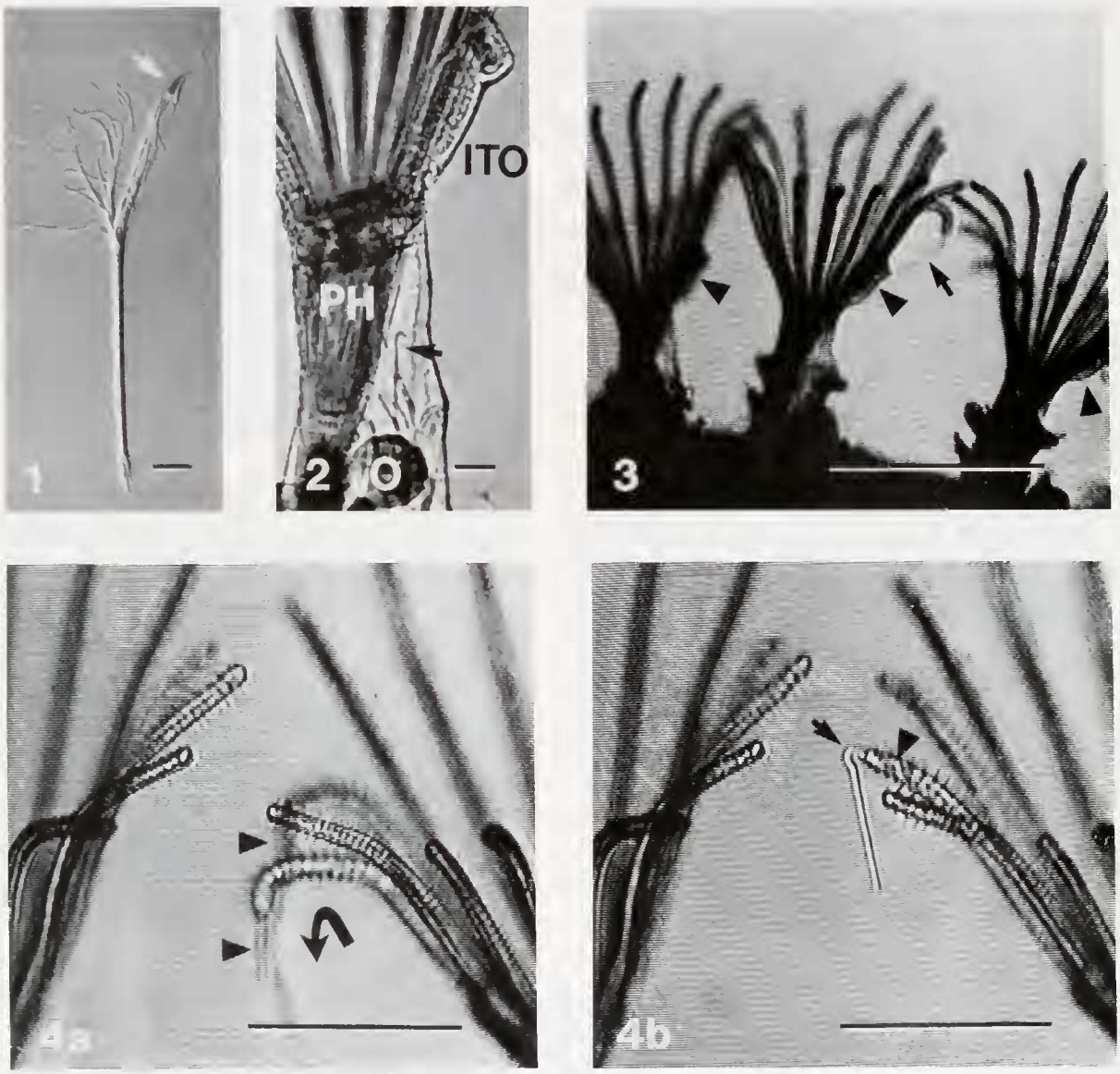
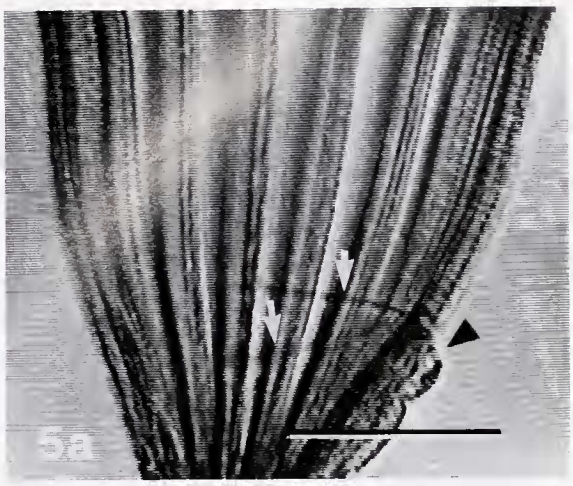


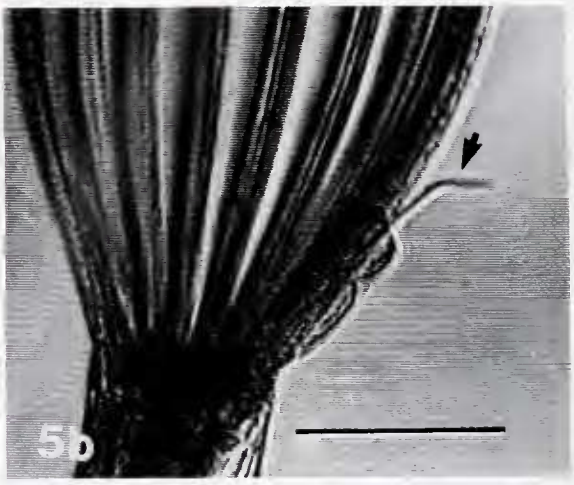
Figure 1. Differential interference contrast image of a partially disassociated spermatozeugma. The arrow indicates the head regions of spermatozoa that have remained joined. The tail region of the spermatozeugma has remained intact. Scale bar = 12 μm.

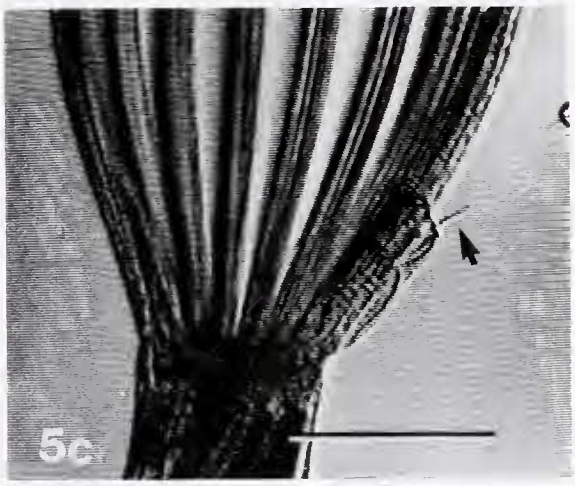
Figure 2. Video image of spermatozeugmata (arrow) and a primary oocyte (O) positioned between the pharynx (PH) and body wall of an extended polypide. The intertentacular organ (ITO) consists of a proximal and distal chamber. Scale bar = 50 μm.

Figure 3. A video image of three extended polypides protruding above the surface of a colony. All three lophophores possess an ITO (arrow heads). A spermatozeugma (arrow) is being spawned through one of the distomedial tentacles of the central lophophore. This tentacle is bent in an abfrontal direction so that the spermatozeugma is released into the exhalent flow of the feeding current passing beneath the canopy of tentacles. Scale bar = 360 μm.

Figure 4. Video images of spermatozeugmata being spawned tail ends first through the tips of the two distomedial tentacles. (a) The tightly bound tail portions of spermatozeugmata (arrow heads) are emerging from the tips of both tentacles. Arrow shows motion of tentacles bending in an abfrontal direction. (b) A spermatozeugma, which has been almost completely discharged, is bent downward at the flexible region (arrow) in the midpiece. The arrow head indicates the portion of the spermatozeugma that is still within the lumen of the tentacle pushing the spermatozeugma out. Scale bars = 150 μm.







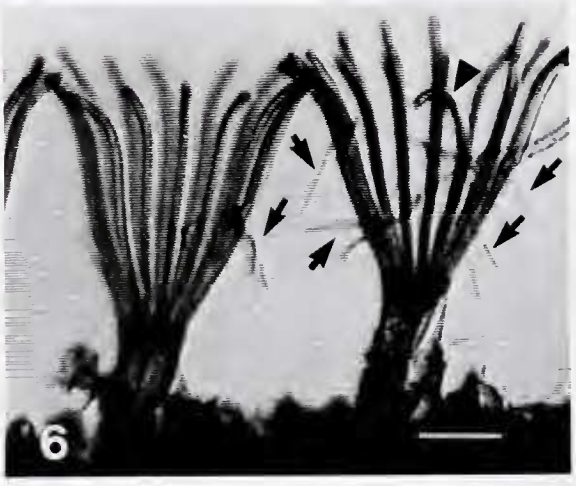


Figure 5. Video images of a spermatozeugma entering the ITO. (a) Two spermatozeugmata (arrows) within the lophophore are oriented so that their heads ends are positioned near the distal opening of the ITO (arrow head). The upper spermatozeugma has its head end inside of the ITO. (b) Thirty-five seconds later this spermatozeugma has been partially drawn into the ITO. The tail end of the spermatozeugma

zooids by dissection to prevent the occurrence of fertilization or activation events during these manipulations. Spawned oocytes were obtained by placing colonies in custard dishes containing 1.0 μm filtered seawater with 0.1 m*M* EDTA. Sexually active colonies transferred from 12–14°C to room temperature usually spawned oocytes within an hour.

Two stains were used in this study to visualize the nuclei within ovarian, coelomic, and spawned oocytes in order to (1) determine the stage and location of oocytes at sperm-egg fusion and (2) describe nuclear events during egg maturation, pronuclear migration, and syngamy. The DNA-specific fluorochrome bisbenzimide H33342 was used at 10 μg/ml by diluting a 100 μg/ml stock in 475 m*M* sodium chloride and 25 m*M* potassium chloride 1:10 with 0.2 μm filtered seawater. Two different staining protocols were followed. Ovaries and oocytes were either (1) fixed for 20 min in 4% formaldehyde buffered with seawater, rinsed thoroughly with 0.2 μm filtered seawater, stained for 5 min with bisbenzimide H33342 (10 μg/ml), and rinsed three times with 0.2 μm filtered seawater; or (2) stained for 10–30 min with bisbenzimide H33342 (10 μg/ml) and rinsed three times with 0.2 μm filtered seawater. Preparations stained with bisbenzimide were mounted for microscopy in 0.2 μm filtered seawater on glass slides with coverslips supported at their corners by plasticene clay "feet." Nuclei stained with bisbenzimide H33342 were visualized using a Zeiss epifluorescent microscope equipped with a 360 nm excitation filter, 395 nm dichroic filter, and 420 nm barrier filter. During prolonged observations, two 50% neutral density filters were placed in the light path to prevent photobleaching of the bisbenzimide fluorescence. Photomicrographs were taken with an Olympus OM-2S camera using 400 ASA Ektachrome slide film.

The second stain used was aceto-orcein. Materials were either (1) fixed for 30 min with 3:1 methanol-acetic acid, stained for 30 min with a 45% solution of aceto-orcein (Humanson, 1979), and rinsed in 20% acetic acid; or (2) fixed for 20 min in 4% formaldehyde buffered with 0.2 *M* phosphate, rinsed thoroughly with Millonig's phosphate buffer rinse (0.2 *M* phosphate, 0.15 *M* NaCl), fixed with 3:1 methanol-acetic acid for 60 min, stained with aceto-orcein for 60 min, and cleared in 30% acetic acid. Aceto-orcein stained preparations were mounted as described above, except that glycerol was used as a mounting medium. These preparations were preserved permanently by ringing the coverslip with nail polish. Materials stained with aceto-orcein were viewed using bright field microscopy, with a green filter (550 nm) inserted into the light path to enhance contrast. To further enhance contrast, differential interference contrast (DIC) microscopy was used in some instances. Photomicrographs were taken with an Olympus OM-2S camera using Panatomic-X film (ASA 32).

Results

Spawning and interzooidal transfer of spermatozeugmata

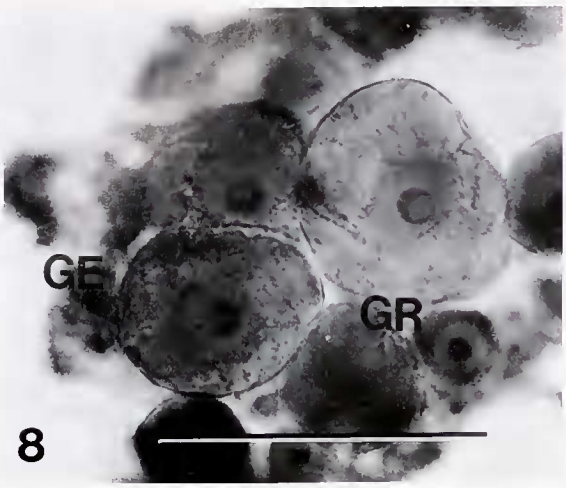
Membranipora membranacea zooids spawn spermatozeugmata through the two distomedial tentacles tail ends first into the exhalent feeding current of the colony. Prior to spawning, spermatozeugmata move freely within the visceral coelom (Fig. 2). Frequently, spermatozeugmata become situated between the body wall and the distomedial side of the pharynx, near the pore that connects the visceral and lophophoral coeloms. However, only those spermatozeugmata oriented with their tail ends toward the pore enter the lophophoral coelom of the distomedial tentacles. As spermatozeugmata move through the tentacles toward the terminal pores, the distomedial tentacles bend in an abfrontal direction (Figs. 3, 4). Consequently, spermatozeugmata emerge from the terminal pores of the tentacles tail ends first into the exhalant feeding current generated by the colony. Spermatozeugmata appear to be pushed through the tentacle lumen by an undulating movement of the midpiece region (Fig. 4b).

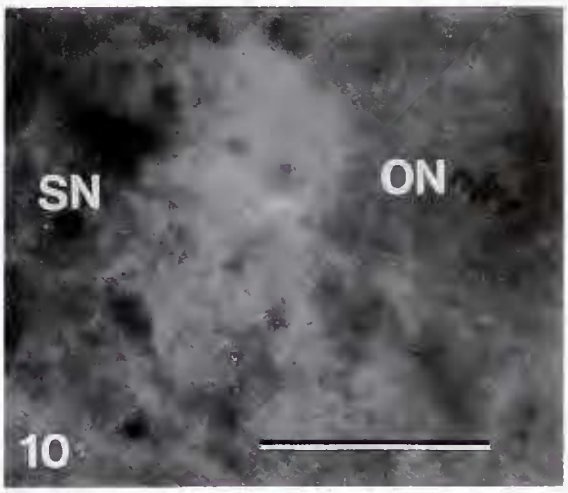
Spermatozeugmata in the water column become entrained in the feeding currents generated by *M. membranacea* colonies. Once drawn into lophophores, strong undulating movements of midpieces begin after spermatozeugmata contact the tentacles. These movements appear to allow some spermatozeugmata to remain within the lophophore long enough to find the distal opening to the ITO. For a spermatozeugma to enter an ITO it must be situated within the lophophore so that its head end emerges between the two distomedial tentacles and in close proximity to the distal pore of the ITO (Fig. 5a). If the head end of a spermatozeugma successfully enters the

Figure 5. (*Continued*) (arrow) is curved away from the lophophore because of the feeding current generated by the tentacles. (c) After 120 seconds, only the posterior portion of the spermatozeugma tail end (arrow) remains outside of the ITO. Scale bars = 100 μm.

Figure 6. Video image of two extended lophophores with attached spermatozeugmata (arrows) but lacking ITOs. One tentacle (arrow head) of the central lophophore is bent in a frontal direction, a movement often used in food capture. Scale bar = 100 μm.

Figure 7. Video image of an ITO (ITO) with a closed distal pore preventing the entrance of spermatozeugmata (arrows). Scale bar = 100 μm.





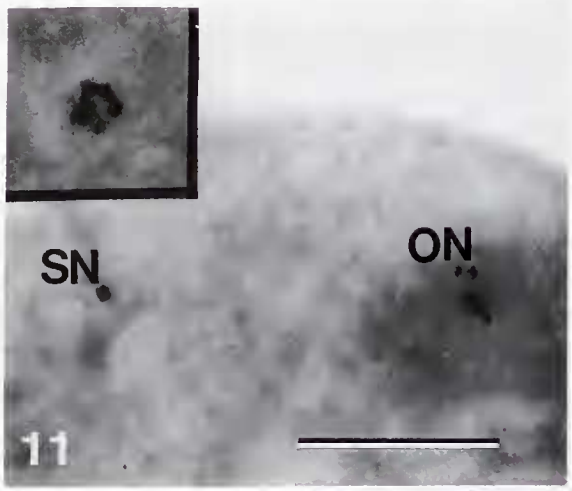


Figure 8. Aceto-orcein stained primary oocytes, containing only an oocyte nucleus as a germinal vesicle, within the growth (GR) and germinal (GE) zones of the ovary. Scale bar = 100 μm.

Figure 9. Single spermatozoa (arrows) present on the surface of follicle cells of an ovary stained with bisbenzimide H33342. Scale bar = 20 μm.

distal pore of the ITO, the undulating movement of the spermatozeugma stops and it is drawn into the ITO (Fig. 5b, c). Spermatozeugmata are drawn completely into ITOs within 2–3 min of first entering the distal pore. Although some of the spermatozeugmata that are drawn into lophophores are eaten, most are rejected as food particles. In several instances, spermatozeugmata became ensnared in the tentacles (Fig. 6). These ensnared spermatozeugmata were never observed to enter an ITO.

The ITO regulates the passage of spermatozeugmata from the external seawater into the ITO. Spermatozeugmata may be prevented from entering an ITO by the closure of the pore located in the distal end of the organ (Fig. 7). However, it is important to note that the ITO does not discriminate between spermatozeugmata produced by genetically identical zooids of the same colony and spermatozeugmata produced by zooids of other colonies. That is, when dishes contain zooids from only one colony, ITOs still permit spawned spermatozeugmata to enter maternal coeloms.

The location of sperm-egg fusion

A comparison of nuclei present in *M. membranacea* ovarian, coelomic, and recently spawned oocytes indicates that sperm fuse with primary oocytes during or shortly after ovulation. All ovarian oocytes contain only an oocyte nucleus, as either a germinal vesicle (Fig. 8) or a set of 12 chromosomes aligned on the first metaphase plate of meiosis, even though spermatozoa are found on the ovarian surface (Fig. 9). In contrast to those in the ovary, coelomic and recently spawned oocytes possess a second nucleus in addition to the oocyte chromosomes aligned on the first meiotic metaphase plate (Figs. 10, 11). This additional nucleus, whose chromatin is organized as chromosomes (Fig. 11), is a partially modified sperm nucleus as confirmed by observations of meiotic divisions and pronuclear migration (see below). Although the sperm nucleus is usually located close to the oolemma, it maintains no specific position with respect to the oocyte nucleus.

The percentage of *M. membranacea* oocytes that are fertilized is very high. For example, 98% of the 396 coelomic and spawned oocytes examined during this study contained a sperm nucleus. In eight instances, background staining may have prevented the observation of a sperm nucleus. On the other hand, the occurrence of polyspermy is low. Only one spawned oocyte possessed more than one sperm nucleus. This polyspermic oocyte contained at least 14 sperm nuclei. Even though the shape of these sperm nuclei was spherical, their chromatin was not always organized as chromosomes (Fig. 12).

Oocyte spawning and egg activation

The ITO mediates the release of oocytes into the ambient seawater (Fig. 13a–e). Oocytes are transferred from the visceral coelom into the ITO via the supraneural pore (Fig. 13a). This transfer takes an average of 42 ± 10 (SD) s (n = 32). During this transfer, oocytes are deformed as they pass through the supraneural pore (Fig. 13b). After being transferred into the ITO, oocytes are propelled toward the distal pore. Oocytes are usually retained within the ITO by the closure of the distal pore (Fig. 13b, c, d). The distal pore may close either before or after an oocyte enters the ITO. Oocytes spend an average of 44 ± 16 SD s (n = 32) in the ITO with the distal pore closed. Following the opening of the distal pore, it takes an average of 17 ± 8 SD s (n = 32) for an oocyte to emerge from the ITO (Fig. 13e). Thus, the total time for an oocyte to pass from the maternal coelom into the external seawater is 104 ± 23 SD s (n = 32), with an average of 61 ± 20 SD s (n = 32) spent within the ITO itself.

At the light microscope level, the two most dramatic morphological modifications of the oocyte during egg activation are a change in cell shape, from discoidal to spherical, and the elevation of a fertilization envelope (Fig. 14). These changes begin only after oocytes are spawned into seawater. The average time for oocytes to begin elevating a fertilization envelope after emerging from the ITO is 180 ± 68 SD s (n = 16). This is true even when oocytes are retained within an ITO for very long periods of time. For example, in one case, the distal sphincter muscle of an ITO remained closed, apparently to prevent the entrance of a spermatozeugma. By this action, two oocytes were restrained from leaving the ITO for more than 4 min, and during this time the oocytes did not change shape, elevate a fertilization envelope, or form polar bodies.

Oocytes are not stimulated to undergo egg activation when they are removed from the maternal coelom and

Figure 10. A coelomic oocyte in polar view stained with aceto-orcein, containing the chromosomes of the sperm nucleus (SN) and oocyte nucleus (ON). Scale bar = 25 μm.

Figure 11. A recently spawned oocyte without a fertilization envelope stained with aceto-orcein. The sperm nucleus (SN) is slightly out of focus. The oocyte nucleus (ON) is a set of chromosomes aligned on the first meiotic metaphase plate. Scale bar = 25 μm. Inset: Chromosomes of the sperm nucleus inside of a recently spawned oocyte.

Figure 12. Four of the fourteen partially modified sperm nuclei (arrows) from a polyspermic spawned oocyte. Scale bar = 20 μm.

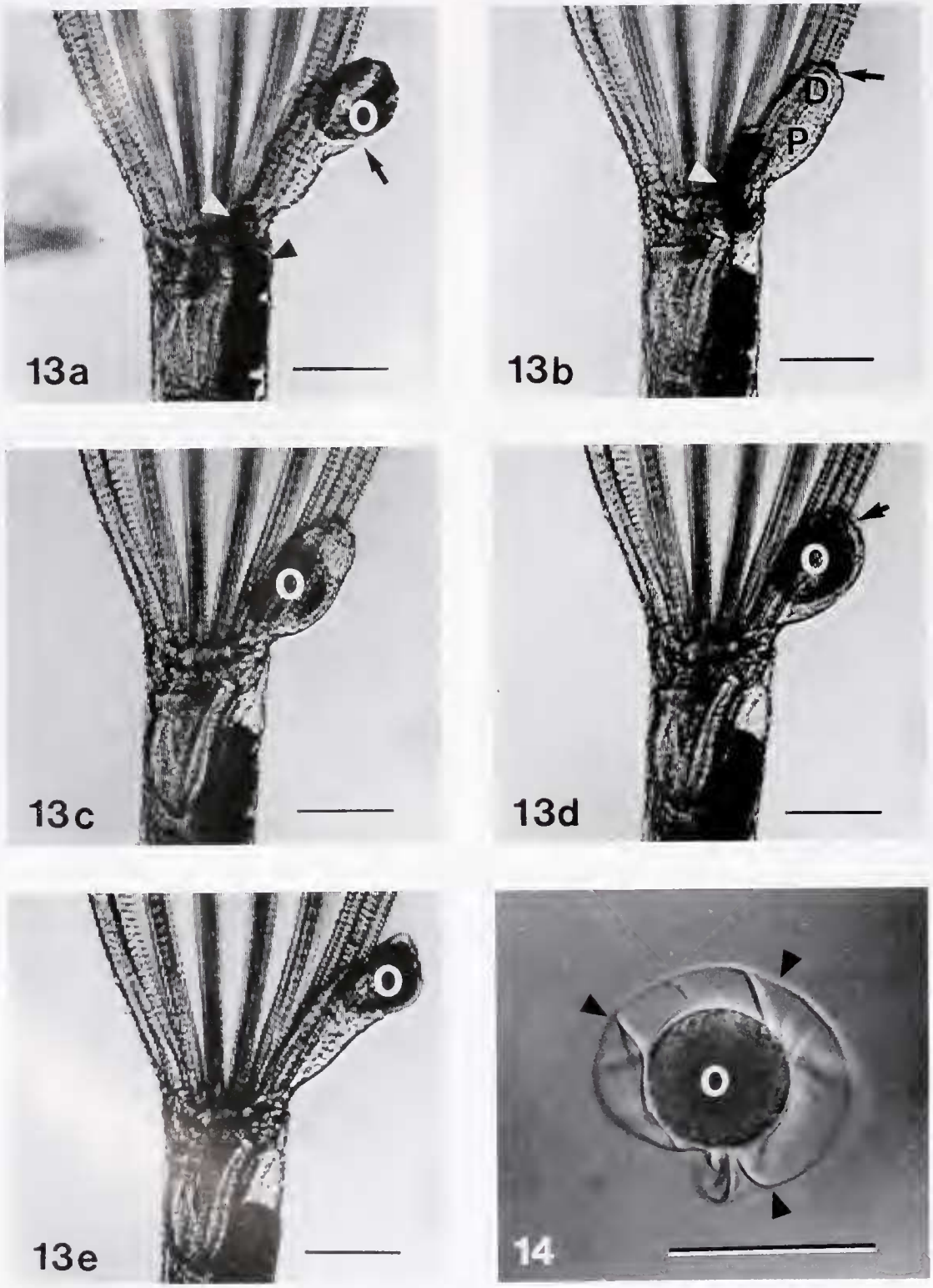


Figure 13. Video images of oocyte spawning in *Membranipora membranacea*. In (a) an oocyte (O) is almost completely discharged from the ITO and the distal pore (arrow) is opened widely. A second oocyte (black arrow head) is about to enter the ITO through the supraneural pore (white arrow head). To the right of the pharynx several other oocytes, which appear as opaque disks, are in line to be spawned (elapsed time = 0). In (b), the distal pore (arrow) of the ITO has closed, while the next oocyte to be spawned is moving

washed with filtered seawater. Only three (6.5%) of the 46 coelomic oocytes so treated formed a fertilization envelope, and none of these three activated oocytes developed. In contrast, 45 (98%) of the 46 oocytes dissected from maternal coeloms and rinsed with filtered seawater containing 0.1 m*M* EDTA underwent egg activation and developed to a swimming gastrula stage.

Egg maturation

The meiotic divisions of the oocyte nucleus begin following the elevation of the fertilization envelope and change in cell shape (Fig. 15). The plane of the meiotic metaphase plate of primary oocytes is parallel to the animal-vegetal axis (Fig. 10). Before the first meiotic division occurs, the meiotic spindle rotates 90° to position a centriole at the animal pole of the zygote. The maturation divisions in *M. membranacea* produce two polar bodies, the first of which does not divide again. Because egg maturation occurs after the elevation of the fertilization envelope, both polar bodies remain within the perivitelline space (Fig. 16).

Pronuclear formation and male pronuclear migration

Following the completion of the meiotic divisions, the chromatin of the female and male pronuclei begins to decondense (Fig. 16) and the male pronucleus starts to migrate toward the female pronucleus. The distance a male pronucleus traverses differs in each zygote, because the entry point of sperm is highly variable. It is not uncommon for a male pronucleus to migrate across the entire diameter of the spherical zygote (about 60 μm). During the migration period, the chromatin of both pronuclei continues to decondense, as indicated by the increasingly diffuse bisbenzimide staining (Fig. 17). After remaining in a highly decondensed state for approximately 20 min, the chromatin of the pronuclei begins to recondense. As this occurs, chromosomes become visible within each of the pronuclei (Fig. 18). By the end of the recondensation period, the pronuclei are ellipsoidal and the bisbenzimide fluorescence is intense (Fig. 19). When the migration of the male pronucleus is completed, the two pronuclei are adjacent to one another, but do not fuse to form a single zygote nucleus.

Syngamy and cleavage

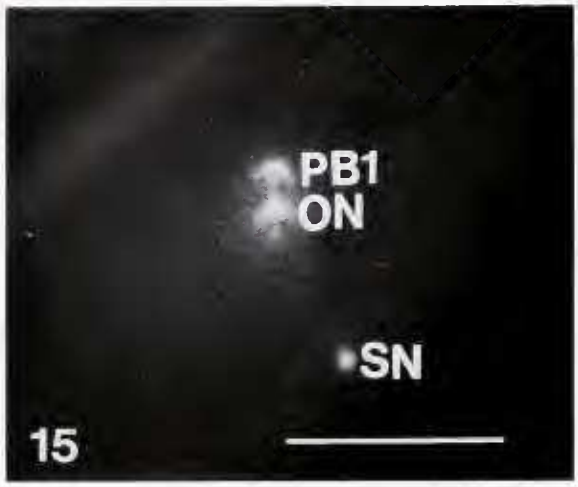
Chromosomes from each pronucleus become aligned on the first mitotic metaphase plate independently of one another. Initially, chromosomes become apparent within each pronucleus. For the three zygotes in which these events were observed, it appears that the chromosomes of the female pronucleus begin to align on the metaphase plate slightly before those of the male pronucleus (Fig. 20). By the end of this phase of fertilization, the chromosomes from both pronuclei are aligned on the same mitotic metaphase plate in preparation for the first cleavage. At temperatures between 12 and 20°C, the first cleavage of zygotes occurs from 60 to 90 min after spawning.

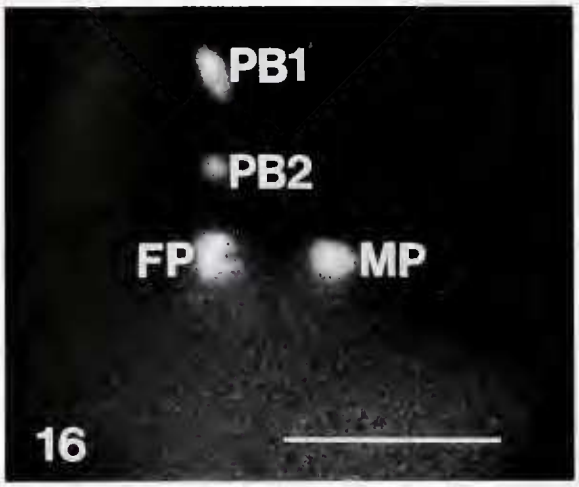
Discussion

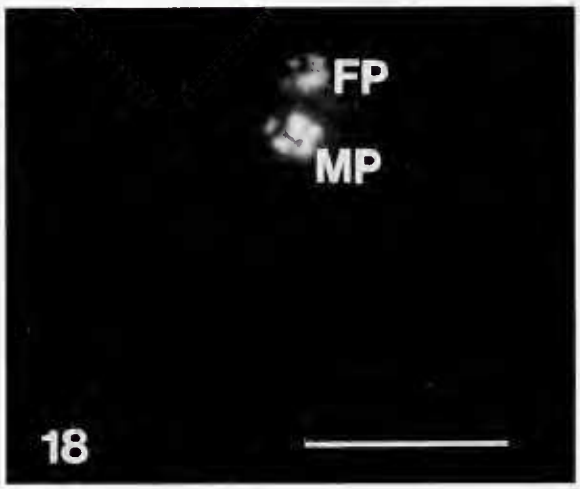
Since Silén (1966) first described the release of sperm by bryozoan zooids two questions have remained unanswered: (1) can spawned sperm enter maternal zooids and (2) are there aspects of sperm and egg spawning that influence whether zygotes are produced by self-fertilization or cross-fertilization? In *Membranipora membranacea*, spawned sperm may enter maternal zooids to fuse with oocytes within the visceral coelom. Spawned *M. membranacea* spermatozeugmata are captured by lophophores of feeding zooids in a manner that is similar to that described for *Electra posidoniae* (Silén, 1966). However, subsequent events may differ between the two species. In *E. posidoniae*, Silén (1966) reported that sperm, adhering to the abfrontal sides of tentacles, detached to swarm around oocytes emerging from the ITO. In *M. membranacea*, some spermatozeugmata become ensnared in the tentacles, but these spermatozeugmata do not detach from the tentacles in response to oocytes being spawned. Instead, spermatozeugmata drawn into *M. membranacea* lophophores swim vigorously trying to maintain a position within the lophophore in order to contact and enter ITOs. Silén (1966) reported the presence of sperm within the ITO of *E. crustulenta*, but did not observe how they entered the ITO. Spermatozeugmata of *M. membranacea* come into contact with ITOs through what appears to be a random search process, and may not be due to chemotaxis. The role of a sperm chemoattractant in directing

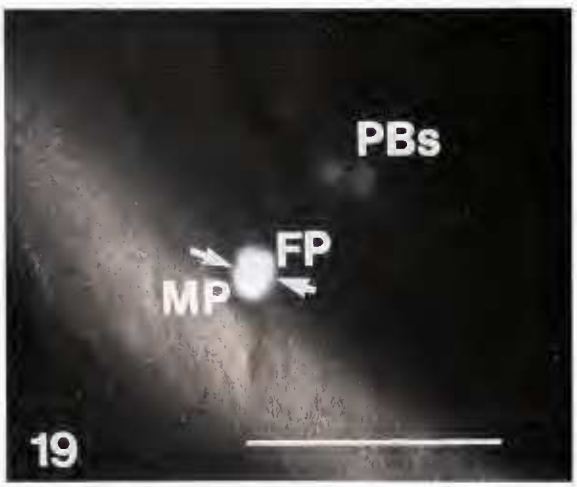
Figure 13. (*Continued*) from the visceral coelom through the supraneural pore (arrow head) into the proximal chamber (P) of the ITO. D denotes the distal chamber of the ITO (elapsed time = 42 s). In (c) the oocyte (O) that is inside the ITO is moving toward the distal pore which is closed (elapsed time = 65 s). In (d) the oocyte (O) is positioned just beneath the distal pore (arrow) of the ITO which remains closed. The closure of the distal pore prevented the release of the oocyte for 25 s after it reached this position (elapsed time = 95 s). In (e) the oocyte (O) is released from the ITO through the pore of the distal chamber (elapsed time = 124 s). Scale bars = 75 μm.

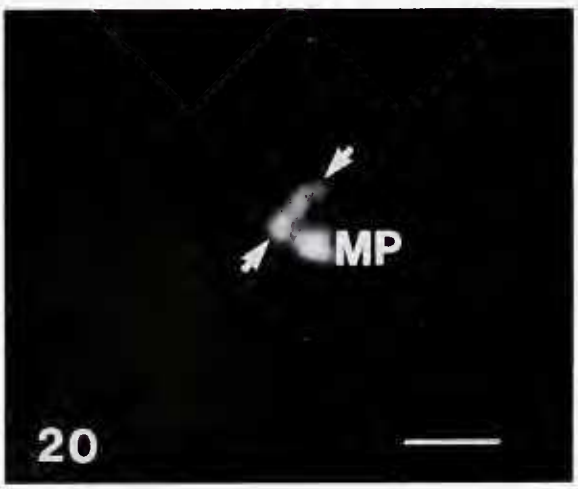
Figure 14. Differential interference contrast image of a spawned oocyte (O) that has an elevated fertilization envelope (arrow heads) and has become spherical. Scale bar = 60 μm.











Figures 15–20. Pronuclear formation and male pronucleus migration in *Membranipora membranacea* zygotes stained with bisbenzimide H33342.

Figure 15. A zygote at first meiotic anaphase shown in side view. The first polar body nucleus (PB1) is located at the animal pole. SN, sperm nucleus; ON, secondary oocyte nucleus. Scale bar = 25 μm.

spermatozeugmata to the ITO seems unlikely because water flow over the ITO is away from the lophophore. Nevertheless, a sperm chemoattractant may be important in directing the movement of spermatozeugmata after initial physical contact with the ITO and in directing sperm to oocytes after ovulation as in other metazoans (Miller, 1985). However, the presence of such chemoattractants in bryozoans is yet to be ascertained.

The release and capture of spermatozeugmata by *M. membranacea* zooids promotes cross-fertilization, but still allows self-fertilization to occur. The ITO in *M. membranacea* does not act as a filter to separate spermatozeugmata produced by genetically identical members of the same colony from those produced by zooids of other colonies. Consequently, both types of sperm cells may enter maternal coeloms to fuse with oocytes. Because *M. membranacea* is not self-sterile (Temkin, 1991), the potential for self-fertilization would increase if sperm did not disperse from paternal/maternal colonies. Silén (1966) suggested that releasing sperm through the tentacle tips would position sperm outside of the paternal zooid's feeding current. But, Silén did not take into account that releasing sperm in this manner might deliver sperm directly into the feeding currents or lophophores of neighboring sister zooids. *M. membranacea* may achieve greater sperm dispersal by bending the distomedial tentacles in an abfrontal direction to release spermatozeugmata into the exhalent flow of the colony's feeding current. In *M. membranacea,* the exhalent current flows out of chimneys or at the edge of colonies (Banta *et al.,* 1974; Lidgard, 1981), decreasing the chances that spermatozeugmata will be retained by the colony that produced them. In fact, electrophoretic studies of protein polymorphism in *M. membranacea* by Thorpe and Beardmore (1981) indicate that the allele frequencies of the few polymorphic loci that they observed were not significantly different from Hardy-Weinburg expections. Still, it is important to remember that *M. membranacea* spawns spermatozeugmata, aggregates of 32 or 64 sperm cells, and even a single spermatozeugma that enters a maternal zooid may have a significant effect on paternity, through either self-fertilization or cross-fertilization.

In *M. membranacea,* sperm-egg fusion is temporally separated from egg activation by the time between ovulation and spawning, a period that Hageman (1983) determined may last as long as four days. In most metazoans, sperm-egg interaction and membrane fusion almost immediately stimulate egg activation, a series of biochemical, physiological, and morphological changes in the egg cell that reinitiate the cell cycle and prevent polyspermy (*e.g.,* Longo, 1987; Epel, 1990). How egg activation is regulated in *M. membranacea* remains to be determined. The fact that egg activation does not begin until after oocytes are released into the ambient seawater indicates that some aspect of the spawning process allows egg activation to occur. However, it is not contact with seawater that initiates egg activation, because oocytes dissected from maternal coeloms and washed with seawater remain unactivated. Coelomic oocytes do have the potential to undergo egg activation as exposing them to 0.1 m*M* EDTA does permit egg activation and normal embryonic development to occur. The mechanism by which EDTA permits the initiation of egg activation in *M. membranacea* oocytes is unclear, and it is not known if EDTA affects oocytes in the same way as the natural stimulus. The low concentration of EDTA used in this study would not be expected to alter levels of Ca^{++} and Mg^{++} in seawater, which are in excess of 10 m*M*. The addition of 0.1 m*M* EDTA to seawater lowers the pH by 0.4 units (7.6 to 7.2), but a comparable decrease in pH alone does not cause *M. membranacea* coelomic oocytes to undergo egg activation (Temkin, unpub.). Two aspects of the spawning process that may affect egg activation are physical stress and exposure to ITO secretions. Oocytes become highly deformed as they pass through the supraneral pore, and this physical stress may initiate egg activation. Alternatively, the proximal chamber of the ITO is glandular (Hageman, 1981) and may secrete a factor that stimulates egg activation. The ITO actively retains *M. membranacea* oocytes for about 1 min. During this time, secretions of the ITO

Figure 16. A zygote shown in side view with the first (PB1) and second (PB2) polar bodies above the female pronucleus (FP) at the animal pole. The chromatin is beginning to decondense in both the female and male (MP) pronuclei. Scale bar = 25 μm.

Figure 17. A zygote viewed from the animal pole, in which the male pronucleus (MP) has migrated toward the female pronucleus (FP). The first polar body is positioned directly above the female pronucleus and thus cannot be seen in this picture. Second polar body nucleus, PB2. Scale bar = 25 μm.

Figure 18. A zygote shown in side view with the female pronucleus (FP) located near the animal pole. The chromosomes of the male (MP) and female pronuclei are apparent as the chromatin of each recondenses. Scale bar = 25 μm.

Figure 19. A zygote shown in polar view in which the male pronucleus (MP) is adjacent to the female pronucleus (FP). The arrows indicate the separation between the two pronuclei. The polar bodies (PBs) which are also fluorescing are out of focus. Scale bar = 25 μm.

Figure 20. In this zygote, the chromosomes of the female pronucleus (arrows) have become aligned on the first mitotic metaphase plate before those of the male pronucleus (MP). Scale bar = 10 μm.

may remove inhibitors from the surface of oocytes either chemically (*e.g.*, chelate) or enzymatically. Another possibility is that an ITO secretion may trigger egg activation through a receptor-ligand interaction. Further studies are required to determine if (1) sperm-egg interaction triggers egg activation, but some inhibitor prevents oocytes from responding to the stimulus until they are spawned or (2) some aspect of the spawning process replaces sperm-egg interaction as the inducer of egg activation.

A pattern of internal sperm-egg fusion and external egg activation may benefit *M. membranacea* zooids in two ways. First, internal sperm-egg fusion allows *M. membranacea* to fertilize nearly 100% of its eggs. This is achieved by maternal zooids capturing spawned spermatozeugmata and concentrating sperm and eggs within the maternal coelom instead of gamete interaction occurring in the water column where dilution factors may severely limit fertilization success (Pennington, 1985; Levitan, 1989; Levitan *et al.*, 1992). Second, the delay between sperm-egg fusion and egg activation allows the passage of pliable oocytes through a much smaller supraneural pore. In almost all species for which egg spawning has been observed, including *M. membranacea*, the passage through the supraneural pore requires oocytes to deform (for a review see Reed, 1991). The deformation may be extreme in species that brood their embryos and produce very large eggs. In these species, oocytes appear "thread-like" as they stream through the supraneural pore into brood chambers (*e.g.*, Silén, 1945; Reed, 1991). It is difficult to believe that once embryos begin to develop and form a hardened fertilization envelope they could undergo such a deformation. It is true that gymnolaemate bryozoan larvae are muscular and are capable of squeezing out of aperatures with slightly smaller diameters than themselves during their release, nevertheless, larvae cannot undergo the dramatic deformation that eggs do during spawning. If intracoelomic egg activation occurred in gymnolaemate bryozoans, it would seem likely that the maternal body wall would need to be ruptured for larvae to be released. Thus, the deformation of pliable oocytes during spawning may play an integral role in preserving the integrity of the maternal body wall and permitting, at the same time, formation of a large egg.

One consequence of extracoelomic egg activation in *M. membranacea* is the prevention of internal brooding. This raises important questions concerning the evolution of brooding patterns in gymnolaemate bryozoans. The majority of gymnolaemate bryozoans brood their embryos at one of four external sites: (1) attached to the external body wall, (2) in the tentacle sheath or vestibule, (3) in embryo sacs, or (4) in ovicells (Hyman, 1959; Ström, 1977; Reed, 1991). Brooding gymnolaemate bryozoan species are believed to have evolved from those that freely spawn their eggs into the ambient seawater such as *M. membranacea* (Jagërsten, 1972; Zimmer and Woollacott, 1977; Strathmann, 1978). It may be that as gymnolaemate species with a lecithotropic larva arose they were constrained from brooding internally by the requirement to spawn oocytes before egg activation could occur. Reports of intraovarian sperm-egg fusion for several species of brooding gymnolaemates (Marcus, 1938, 1941; Correa, 1948; Mawatari, 1952; and Dyrynda and King, 1983) suggest that internal sperm-egg fusion may be typical for gymnolaemate bryozoans. However, a complete evaluation of this proposal requires further study of sperm-egg fusion and egg activation in more brooding and non-brooding species.

In summary, this investigation provides the most complete description of the fertilization process for any gymnolaemate bryozoan to date. Except for the temporal separation between sperm-egg fusion and egg activation, *M. membranacea* has an *Ascaris*-type fertilization process. The *Ascaris*-type fertilization process is characterized by (1) overlap of late oogenic and early fertilization events and (2) syngamy at the onset of the first mitotic division of the zygote, without the fusion of male and female pronuclei to form a zygote nucleus (Wilson, 1925; Longo, 1987). The *Ascaris*-type fertilization process is typical of species in which sperm fuse with primary or secondary oocytes. In *M. membranacea*, primary oocytes arrested at first meiotic metaphase fuse with a single sperm cell during or shortly after ovulation, but do not undergo egg activation until they are spawned into the ambient seawater. Nevertheless, internal sperm-egg fusion in *M. membranacea* does not preclude the possibility for cross-fertilization because spawned spermatozeugmata may enter the maternal coelom through the ITO.

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